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Cytokine regulation on the synthesis of nitric oxide *in vivo* by chronically infected human polymorphonuclear leucocytes

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SUMMARY

To determine if nitric oxide (NO) is produced by chronically infected human polymorphonuclear leucocytes (PMNs) *in vivo*, inflamed exudates (periapical exudates: PE) collected from periapical periodontitis patients were examined. Cell-free supernatants and cells were separated by centrifugation. Significant levels of nitrite concentrations were observed in the supernatants. The production of inducible NO synthase (iNOS) in highly purified PMNs derived from PEs was then immunocytochemically determined using rabbit anti-human iNOS antiserum. *In vitro*, human peripheral blood PMNs (PB-PMNs) isolated from patients were cultured with a combination of *Esherichia coli*–lipopolysaccharide (LPS), recombinant human interferon-γ (rhIFN-γ) and/or interleukin-1β (rhIL-1β). The stimulated PB-PMNs showed steady-state levels of nitrite. The stimulation of LPS, rhIFN-γ and rhIL-1β showed more NO induction than that of LPS with either IFN-γ or IL-1β, suggesting the synergistic effects of cytokines. Cryostat sections of surgically removed periapical tissues were also immunohistochemically examined for iNOS, IFN-γ and IL-1β. Two-colour immunohistochemistry revealed the interaction of iNOS-producing PMNs and IFN-γ- or IL-1β-producing mononuclear cells. On the basis of these data, we concluded that with the stimulation of inflammatory cytokines derived from mononuclear cells, PMNs can spontaneously produce NO at the site of chronic infection. The present studies are consistent with a hypothesis suggesting that PMNs could be regulated and delicately balanced to produce NO by mononuclear cell-derived cytokines *in vivo*. NO-producing cells may play a pivotal role in chronic inflammation.

INTRODUCTION

Recently, nitric oxide (NO) has been identified as an endothelium-derived relaxing factor, which regulates blood pressure, and characterized to be a gaseous free radical.[1–3] It is known to have a variety of biological activities, and the roles of NO have been proposed in the decrease of neutrophil chemotaxis,[4] in the inhibition of neutrophil adhesion to endothelium,[5] or in the dysfunction of lysosomal enzyme release.[6] NO also up-regulates tumour necrosis factor-α (TNF-α) production by polymorphonuclear leucocytes (PMNs)[7] and can be the regulator of inflammation and the modulator of bone remodelling.

It has been shown that NO is produced by a variety of rodent cells;[8,9] however, *in-vivo* aspects of human cells, especially PMNs, are not fully established for NO production. For example, Klebanoff *et al.*[10] have shown that a combination of phorbol 12-myristate 13-acetate (PMA), catalase and NaN_3 exhibits the increase of NO secretion by human peripheral blood (PB) PMNs. Several researchers have also raised the possibility that inflammatory cytokines such as interleukin-1β (IL-1β), TNF-α or interferon-γ (IFN-γ) with bacterial lipopolysaccharide (LPS) can induce NO secretion by human PB-PMNs *in vitro*.[11] On the other hand, others have demonstrated that human PMNs produce significantly low levels of NO in response to LPS and IFN-γ and may lack the ability to secrete detectable NO.[12] Most studies have been performed on animal models[8,9,13] or *in vitro* experiments.[10–12] Thus, the ability to produce NO *in vivo* by human PMNs has remained controversial.

Periapical periodontitis is a chronic infectious disease, and many kind of bacteria in the oral cavity are involved. Granuloma formation and bone resorption around the apex of teeth are typical pathological and clinical features. Inflamed exudates (periapical exudates: PEs) derived from periapical lesions are rich in PMNs and would involve variable subcellular information. Recently, Sakurai *et al.*[14] have shown that synovial cells isolated from rheumatoid arthritis patients produce significant levels of NO. Thus, NO may be involved in tissue

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damage in chronic inflammation. To know the direct evidence of NO production by PMNs, it is essential to analyse inflamed exudates. The purpose of the present study was to elucidate whether PE-PMNs have a capability to produce NO *in vivo* and possible interaction of cytokine-producing cells.

MATERIALS AND METHODS

Patients

In the patients who were diagnosed with periapical periodontitis based on clinical criteria (pain, swelling of gingivae around the apex of a tooth, X-ray, etc.), 30 patients (17 males and 13 females) ranging in age from 28 to 56 years were subjected. Apparent radiolucency (15–25 mm in size) was seen around the apex of subjected teeth in all patients. No systemic disease was observed in any patients, and antibiotics have not been taken during the previous 6 months. Written consent was obtained from all patients prior to the collection of samples.

Sample collection and PE-PMN preparation

Inflamed PEs were collected through root canals of patients ($n=20$) directly using sterile microdispender replacement tubes (Drumond Scientific Co., Broomall, PA). To avoid contamination by saliva, the sampling area was isolated with a rubber dam dry field technique. The collected exudates were then centrifuged to separate cells and cell-free supernatants. The composition of the cells were PMNs (96·2%), lymphocytes and macrophages as determined by haematoxylin–eosin (H–E) and non-specific esterase stains using cytospin specimens. The PE-PMNs were then purified by Ficoll–Hypaque (Pharmacia LKB Biotechnology, Uppsala, Sweden) gradient centrifugation. The number of collected PMNs was $\approx 1 \times 10^5$ and the purity of the PMNs was $\approx$99·1%.

Inflamed tissues of periapical lesions were obtained from patients ($n=10$) at the time of surgical treatments for the removal of pathological tissues. Immediately, the tissues were cut into two pieces. Paraffin and cryostat sections were then prepared as 5 μm-thickness and fixed with acetone or 4% paraformaldehyde in phosphate-buffered saline (PFA/PBS), respectively.

Quantification of nitrite (NO_2^-) concentration

The cell-free supernatants of PEs collected above were analysed in duplicate for the determination of nitrite concentration, which is the stable end products of NO oxidation, as previously described.[15] NO measuring kit (Nitric oxide colorometric assay, Boehringer Mannheim, Mannheim, Germany) was utilized. In brief, nitrate reductase with potassium phosphate buffer (pH 7·5) was incubated with the supernatants at room temperature for 30 min. An equal volume of Griess reagent (1% sulphanilamide, 0·1% naphthylethylene-diamine dihydrochloride, 2·5% phosphoric acid) was then added to the supernatants and incubated at room temperature for 5 min. Absorbance values were determined using a spectrophotometer at 550 nm. The nitrite concentrations were then determined using a standard curve generated with serial dilution of sodium nitrite. Reaction mixtures without supernatants were also analysed in parallel as negative controls.

Immunocytochemistry

The cytospin specimens of the highly purified PE-PMNs obtained above were prepared using a cytocentrifuge and fixed with 4% PFA/PBS. iNOS production was then examined by avidin-biotin complex (ABC) method using human iNOS specific antibodies (Abs). In brief, the samples were blocked with goat normal serum for 20 min at room temperature and were then incubated for 1 hr with affinity-purified anti-human iNOS antiserum (generated by immunization of rabbits with a synthetic NH_2-terminal peptide of human iNOS: Met-Ala-Cys-Pro-Trp-Lys-Phe-Leu-Phe-Lys-Val-Lys-Ser-Tyr) or normal rabbit immunoglobulin G (IgG) Abs as a negative control. The slides were washed with PBS, and biotinylated anti-rabbit IgG Abs were then incubated for 1 hr, followed by the incubation with avidin glucose oxidase (Vector Laboratories, Inc., CA). After final wash, iNOS positive cells were developed with glucose oxidase substrate (tetranitroblue tetrazolium, Vector Laboratories) and were counterstained with methyl green. iNOS positive cells were then carefully examined using a light microscope.

Induction of NO synthesis in PB-PMNs

Heparinized peripheral blood was intravenously collected from five patients, and PB-PMNs were isolated using Ficoll–Hypaque gradient centrifugation. Red blood cells in the cell suspension were then lysed with 0·87% NH_4Cl. Purity was determined by H–E and non-specific esterase stains. These cells were resuspended in minimum essential medium containing 10% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 mg/ml). The isolated PB-PMNs were then cultured in 24-well culture plates (Nunc, Roskilde, Denmark) with or without a combination of LPS (*Escherichia coli*, 0111:B4, 10 μg/ml, Difco, Detroit, MI), recombinant human IL-1β (rhIL-1β, 2 ng/ml, Genzyme, Cambridge, MA) and human IFN-γ (rhIFN-γ, 10 ng/ml, Genzyme) in a humidified 5% CO_2-in-air incubator at 37° for 2 or 20 hr. The number of cultured PB-PMNs in each well was 2×10^6 cells and the volume of culture medium was 500 μl. Viability was determined by ethidium bromide–acridine orange solution, before and after stimulation. At each culture period, culture supernatants were harvested and nitrite concentrations were determined immediately, as previously described. For the neutralization of cytokine stimulation, rhIL-1β (2 ng/ml) or rhIFN-γ (10 ng/ml) was preincubated for 1 hr with anti-human IL-1β (20 ng/ml, Oncogene Science, Uniondale, NY) or anti-human IFN-γ (100 ng/ml, PeproTech, London, UK) polyclonal Abs, respectively. LPS (10 μg/ml) was also preincubated with polymyxin B (10 μg/ml) at 37° for 30 min. PB-PMNs were cultured for 2 or 20 hr in the absence or presence of the neutralized LPS, rhIL-1β and rhIFN-γ. Nitrite levels were then determined.

Immunohistochemistry

Immunohistochemical analysis for human iNOS was performed using frozen tissue sections of 10 patients, which were prepared as 5 μm-thickness. The staining procedures were followed as described in Immunocytochemistry. The specimens were finally examined using a light microscope.

Immunofluorescence microscopic analysis

The localization of IL-1β- or IFN-γ-producing cells in the tissues were analysed by two-colour immunohistochemistry. iNOS-immunohistochemistry and indirect immunofluorescence microscopy for IL-1β or IFN-γ were performed on the same specimens. In brief, cryostat sections were blocked, and

anti-human iNOS Abs with either anti-human IL-1β (Oncogene Science) or IFN-γ monoclonal Abs (PharMingen, San Diego, CA) were co-incubated on the specimens. iNOS-positive cells were developed as previously described, and cytokine production was then detected using RITC conjugated rabbit anti-mouse IgG Abs (Tago Immunologicals, Camarillo, CA) for IL-1β or fluorescein isothiocyanate (FITC) conjugated goat anti-mouse IgG Abs (Tago) for IFN-γ. The specimens were mounted with aqueous mounting medium (fluoromount-G, Southern Biotechnology, Birmingham, AL) and then examined using a fluorescent microscope.

Statistics

Statistical analysis was assessed by student *t*-test and $P \leq 0{\cdot}01$ was taken as statistically significant.

RESULTS

Nitrite concentration in PEs

The nitrite concentration in the cell free supernatants of PEs were measured using Griess reagent. Low, but steady-state levels of nitrite were shown (Fig. 1). The amounts of nitrite were widely ranged from 0·44–6·54 μM and only one sample showed a high level of nitrite concentrations (32·25 μM). The mean value was 4·84 μM.

Detection of iNOS protein in inflamed PE-PMNs

Cytospin specimens of highly purified PE-PMNs were evaluated by H–E stain (Fig. 2a) and then analysed immunocytochemically using anti-human iNOS antiserum. The PE-PMNs showed significant levels of immunoreactivity to the iNOS in their cytoplasms (Fig. 2b). The average of iNOS positive PMNs was ≈91%, and the proportion did not vary with individuals (data not shown). Negative controls never showed a positive reaction with iNOS antiserum (Fig. 2c).

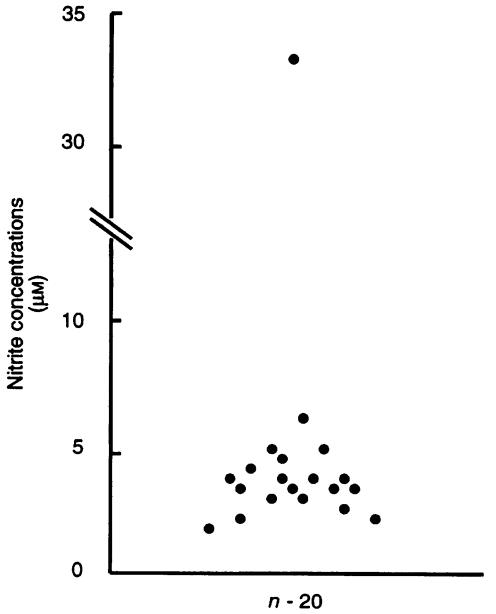

Figure 1. Nitrite concentrations in the cell-free supernatants of PEs. Griess reagents were mixed with the supernatants, and absorbance values at 550 nm were obtained using a spectrophotometer. Nitrite concentrations were shown as μM.

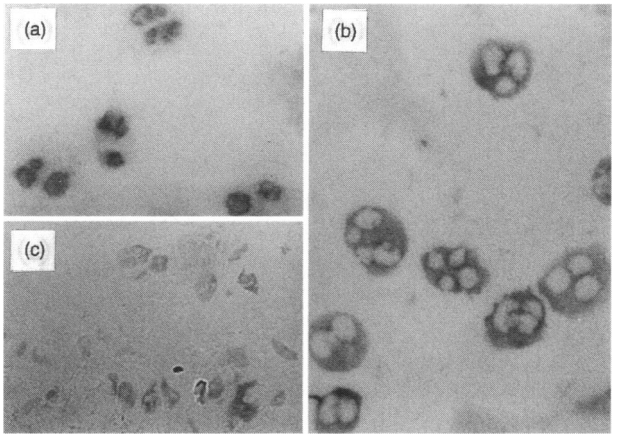

Figure 2. Detection of iNOS reactivity in highly purified PE-PMNs. (a) Haematoxylin-eosin staining. (b) Immunocytochemistry using human iNOS antiserum. Counterstained with methyl green. Immunoreactivity to iNOS was exhibited in the cytoplasm of PE-PMNs. (c) Negative controls without iNOS antiserum. Original magnification is ×400.

Induction of NO production in PB-PMNs

PB-PMNs isolated from patients were stimulated with a combination of LPS, rhIL-1β or rhIFN-γ (Fig. 3). At 20-hr stimulation, a mixture of LPS, rhIL-1β and rhIFN-γ induced 224·8 ± 26·0 μM of nitrite in 1×10^6 PMNs and exhibited more significant NO induction than LPS with either rhIL-1β (nitrite levels; 90·2 ± 32·1 μM/1 × 10^6cells) or rhIFN-γ (nitrite levels; 158·4 ± t20·4 μM/1 × 10^6cells), suggesting the possibility of synergistic effects of these cytokines on NO synthesis ($P \leq 0{\cdot}01$). The amounts of nitrite concentrations with the stimulation for 20 hr showed higher levels in comparison with 2-hr stimulation.

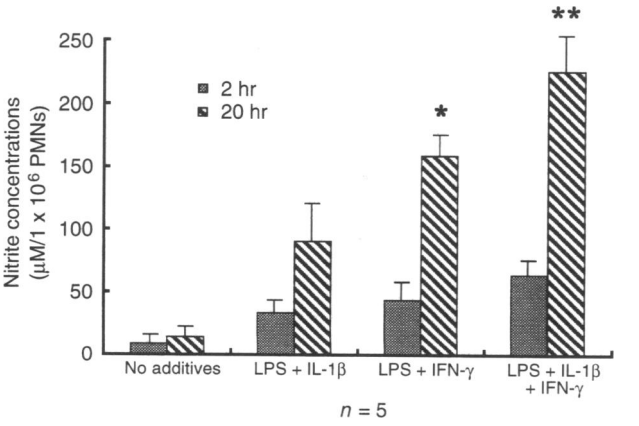

Figure 3. NO production by stimulated peripheral blood PMNs isolated from patients. PB-PMNs were cultured with a combination of LPS, rhIL-1β and/or rhIFN-γ for 2 or 20 hr. Nitrite concentrations were then determined. The values shown here were calculated to μM/1 × 10^6 PMNs. *Significant difference ($P<0{\cdot}01$) compared to the stimulation with LPS + rhIL-1β; **compared to LPS + rhIL-1β and LPS + rhIFN-γ.

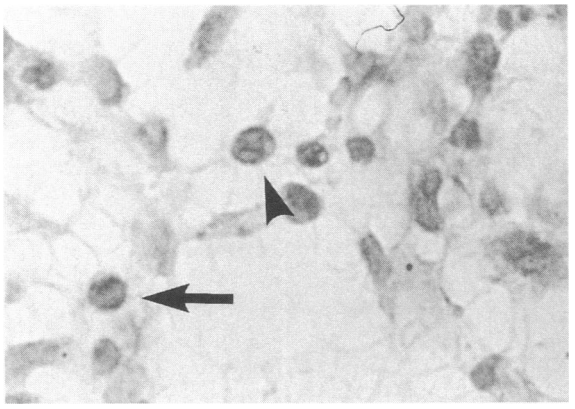

Figure 4. Immunohistochemistry of iNOS production in inflamed tissues. Cryostat sections were stained with antiserum against human iNOS. PMNs (arrowheads), as well as mononuclear cells (arrows) showed immunoreactivity to iNOS. Original magnification is ×200.

Nitrite levels of negative controls at 2- or 20-hr incubation were 8·91 or 14·6 μM.

LPS, rhIL-1β and rhIFN-γ neutralized with polymyxin B, anti-human IL-1β and IFN-γ polyclonal Abs, respectively, exhibited less NO production by PB-PMNs (data not shown).

Immunohistochemical analysis of granuloma tissues

Surgically removed periapical tissues were evaluated by H–E stain and determined as granuloma tissues. The specimens were then immunohistochemically examined for iNOS. PMNs, as well as mononuclear cells exhibited immunoreactivity to iNOS in their cytoplasms (Fig. 4). Endothelial cells and fibroblasts were significantly reacted with iNOS Abs (data not shown). Interestingly, cells adjacent to blood vessels showed significant iNOS synthesis, whereas cells apart from the blood vessels showed weak or no iNOS synthesis. In addition, some lymphocytes showed iNOS synthesis, but some did not.

Indirect immunofluorescence microscopy

Two-colour immunohistochemistry was performed using iNOS and either IL-1β or IFN-γ Abs, as shown in Figs 5 and 6. Mononuclear cells were stained with monoclonal Abs against IL-1β or IFN-γ. IL-1β-producing cells were morphologically determined as macrophages and lymphocytes, whereas IFN-γ-producing cells were lymphocytes. The data revealed that iNOS-producing cells (Figs 5a and 6a) have a capability to produce IL-1β (Fig. 5b) or IFN-γ (Fig. 6b), as shown with arrows. However, some cells with immunoreactivity to iNOS did not show those cytokine production (arrowheads in Figs 5a and 6a). A negative control using normal rabbit IgG Abs never showed positive staining with any cells (data not shown).

DISCUSSION

It has been demonstrated that the suppressive effects of activated macrophages on the proliferative responses of lymphocytes to mitogens or antigens can be attributed in part to NO.[16] In addition, NO induces the killing of cells by means of promoting apoptosis,[17] causing intracellular iron loss,[18] inhibition of mitochondrial respiration[19] and inhibition of

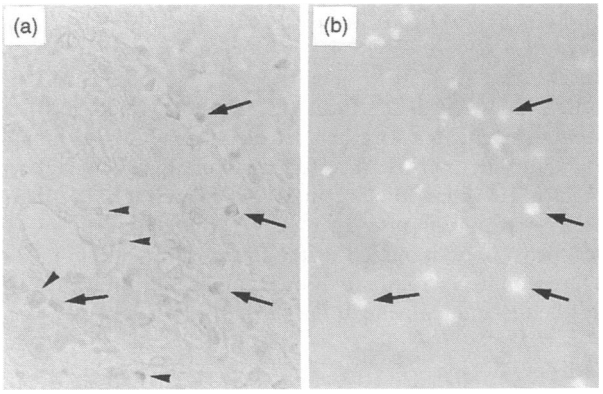

Figure 5. Two-colour immunohistochemistry. iNOS-positive cells were developed (a), and IL-1β-producing cells were then detected using human IL-1β monoclonal Abs, followed by the incubation with RITC conjugated rabbit anti-mouse IgG Abs (b). IL-1β was significantly produced by macrophages or lymphocytes. Some iNOS-producing cells synthesized IL-1β (arrows), but some did not (arrowheads). Original magnification is ×100.

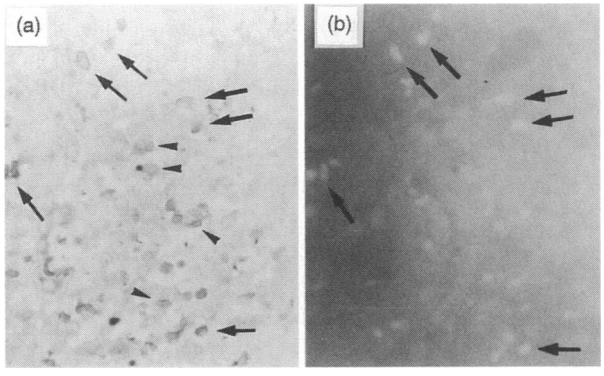

Figure 6. Two-colour immunohistochemistry. iNOS-positive cells were developed (a), and IFN-γ-producing cells were then detected using human IFN-γ monoclonal Abs, followed by the incubation with FITC conjugated goat anti-mouse IgG Abs (b). IFN-γ was significantly produced by lymphocytes. Some iNOS-producing cells synthesized IFN-γ (arrows), but some did not (arrowheads). Original magnification is ×100.

DNA synthesis.[20] Therefore, it is thought to modulate inflammation and has been introduced in substantial tissue destruction or in metabolic dysfunction.[21,22]

There have been a number of publications studying NO secretion; however, most studies have examined animal models or human peripheral blood cells with stimulation. Animal studies have been especially well performed. Because the amount of NO secretion by human PB-PMNs is significantly low, it is not clearly defined whether PMNs can produce NO or not.

In this study, inflamed exudates collected from the sites of bone resorption with chronic infection were analysed. Steady-state levels of nitrite in the exudates were exhibited using Griess reagent, as well as the nitrite levels in synovial fluid isolated from rheumatoid arthritis patients.[23] NO production has been shown to be regulated by the various isoforms of NOS.[24,25] Especially, iNOS is more remarkable than a constitutive form of NOS, because it is significantly synthesized with the appropriate stimulation at the site of inflammat-

ion. Therefore, iNOS production by PMNs isolated from PEs was also carefully examined immunocytochemically. Immunoreactive iNOS protein was substantially detected in the cytoplasm of the PMNs. Thus, this NO production would be derived from PE-PMNs. Remarkably, the proportion of iNOS-positive staining among subjected patients was similar, although the amount of the nitrite production in PEs was widely ranged from 0·54 μM. The reason may be because highly purified PMNs were analysed in this study and the other cells could not affect.

One subject exhibited the excessive amount of nitrite (32·25 μM). Chronic inflammation occasionally changes its stage to acute phase. Therefore, the reason may be explained by the possibility that a large number of NO-producing PMNs could be present because of a change to acute phase at the time of sample collection.

Because PEs were characterized not only by PE-PMNs but also by cells in periapical tissues, a number of infiltrating cells in the tissues could also contribute to NO secretion. Therefore, frozen tissue sections of periapical lesions were then analysed for immunoreactive iNOS protein. In periapical inflamed tissues, endothelial cells and fibroblasts were significantly stained with iNOS antiserum. Inflammatory cells including PMNs, macrophages and lymphocytes also showed iNOS reactivity. Interestingly, some lymphocytes showed iNOS synthesis, but some did not. In terms of this finding, we hypothesized that a part of inflammatory cells are induced for iNOS synthesis with appropriate stimulation. However, there is increasing evidence to indicate that T helper type 1 (Th1) cells can synthesise iNOS but Th2 cells can not.[26] Therefore, the another reason would be explained by the hypothesis suggesting that iNOS negative mononuclear cells in periapical lesions could be T helper type 2 (Th2)-type lymphocytes.

The localization of IL-1β- or IFN-γ-producing cells in the inflamed tissues were analysed. IL-1β-producing cells were mostly macrophages and lymphocytes, and IFN-γ-producing cells were lymphocytes. Furthermore, two-colour immunohistochemistry using iNOS and either IL-1β or IFN-γ Abs demonstrated that these types of cells also have a capability to produce iNOS. Hence, autocrine and paracrine effects of cytokines by mononuclear cells could be suggested.

We prepared PB-PMNs from periodontitis patients and then cultured them with LPS, IL-1β and/or IFN-γ using 24-well culture plates. To obtain high concentrations of nitrite production, 2×10^6 PMNs were resuspended with 500-μl culture medium in each well. This cell number would be the maximum so as not to cause cell death in the association with cell-to-cell communication. NO production with single cytokine stimulation was significantly less amount than with the stimulation of both ($P < 0{\cdot}01$). The data suggested that IL-1β has synergistic interactions with IFN-γ for iNOS induction. In our preliminary studies for the measurement of nitrite, PB-PMNs isolated from periodontally healthy volunteers were also analysed and the nitrite levels between periodontal patients and healthy individuals did not vary.

We have previously demonstrated that periapical exudates isolated from periapical periodontitis patients involve inflammatory cytokines such as IL-1α, IL-1β, TNF-α and IL-6, and it has been hypothesized that these cytokines are derived from cells not only in the inflamed exudates but also in tissues around the apex of the tooth.[27] On the basis of our previous data and ample published evidence, we assumed that inflamed PMNs in periapical lesions could synthesize iNOS in response to LPS and cytokines *in vivo*. Present studies can strongly support this possibility with the results in *in vivo* and *in vitro* analyses of clinical samples.

Cytokines can modulate many aspects of cellular growth, differentiation and activation. They might be associated with the initiation of inflammation. On the other hand, cytokine regulation could also be important in controlling the activities of iNOS. Because significant levels of NO must be spontaneously produced for prolonged periods in inflamed sites, it causes an increase in tissue damage and could be associated with chronic inflammation.

Interestingly, NO-knockout mice exhibited an enhanced Th1, but an impaired Th2 immune response with antigenic stimulation.[28] More recently, it has been demonstrated that NO inhibits the secretion of Th1 and Th2 cytokines in activated human T cells.[29] In agreement with these observation and our present study, we speculate that human chronic inflammation could be precisely controlled by NO. It has been demonstrated that an L-arginine analogue can inhibit NO synthesis.[30,31] These concepts could lead us to improve pharmacological therapy of periapical periodontitis. Further animal studies must be needed to elucidate this hypothesis.

Because of its short half-life, NO may not diffuse far from the site of NO production in inflamed tissues. NO should be synthesized locally to be involved directly in the pathology and biochemistry of periapical lesions. Therefore, it is consistent with an hypothesis suggesting that NO producing cells could control immune cell responses, contribute to comprehensive tissue destruction and play a pivotal role in inflamed tissues. In the event, we concluded that periapical lesions, caused by chronic inflammation with bacterial infection, can be regulated and delicately balanced by the biological activities of NO with respect to cytokines derived from mononuclear cells *in vivo*.

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